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Infection With GB Virus C/Hepatitis G Virus in Brazilian Hemodialysis and Hepatitis Patients and Asymptomatic Individuals

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Recently, sequences from a novel human flavivirus, termed GB virus C (GBV-C) or hepatitis G virus (HGV), have been identified in serum from patients with cryptogenic hepatitis and others. Sera from 116 patients with different clinical backgrounds were tested for the presence of GBV-C/HGV RNA by a reverse transcription-polymerase chain reaction with primers from the nonstructural (NS) 5 region. Ten (15%) patients on maintenance hemodialysis and 5 (19%) non A-C hepatitis patients were GBV-C/HGV RNA positive, along with one patient with chronic hepatitis B, one patient with chronic hepatitis C, and two asymptomatic individuals. Sequence comparison within 354 base pairs in the NS5 region showed homology rates varying from 87% to 97% among five Brazilian isolates, and from 86% to 93% between Brazilian strains and GBV-C/HGV isolates from other countries previously sequenced. Homology rates were higher at the amino acid level since most substitutions occurred at the third nucleotide position of codons without changing the codon meaning. ***J. Med. Virol. 52:61–67, 1997.***

KEY WORDS: polymerase chain reaction; nucleotide sequencing; nonstructural 5 region; phylogenetic analysis

INTRODUCTION

Recently, three new members of the *Flaviviridae* have been identified [Simons et al., 1995a,b]. The isolation of the GB viruses A and B (GBV-A and GBV-B) as novel flavivirus agents in tamarins infected with serum from a surgeon with acute hepatitis [Simons et al., 1995b] has allowed the identification of another virus, GBV-C, from a human specimen [Simons et al., 1995a]. Independently, an RNA virus, designated hepatitis G virus (HGV), was identified from the plasma of a patient with chronic hepatitis [Linnen et al., 1996]. The two viruses were closely related to each other and distantly related to hepatitis C virus (HCV), GBV-A, and GBV-B. Alignment of the nucleotide sequence of GBV-C with that of HGV has revealed a homology of 85% (95% at the amino acid level) and GBV-C and HGV appear to be two isolates of the same virus [Zuckerman, 1996], termed here GBV-C/HGV.

The viral genome is a single-stranded RNA of approximately 9.4 kb that encodes a putative single large polyprotein of 2873 to 2910 amino acids in which, similarly to HCV, the structural proteins are positioned at the N-terminal end, with the non-structural proteins NS2, NS3, NS4, NS5A, and NS5B located at the C-terminal end. Sequences of GBV-C/HGV can be detected in human serum after amplification by reverse transcription-polymerase chain reaction (RT-PCR). RNA of this virus has been found in 0.9 to 2% of voluntary blood donors in the United States [Linnen et al., 1996], Italy [Fiordalisi et al., 1996], and Japan [Masuko et al., 1996]. The virus has also been detected in plasma pools and in intravenous immunoglobulin [Nübling and Löwer, 1996]. A high prevalence of GBV-C/HGV RNA has been found in patients with hepatitis of unknown etiology, i.e., non A-E hepatitis [Fiordalisi et al., 1996], and in subjects with frequent parenteral exposure, including intravenous drug users [Aikawa et al., 1996; Linnen et al., 1996], patients on hemodialysis [De Lamballerie et al., 1996; Tsuda et al., 1996], and patients with hemophilia [Linnen et al., 1996]. The virus is known to be transmitted parenterally [Linnen et al., 1996; Schmidt et al., 1996] and to be able to persist in the same patient for many years [Masuko et al., 1996]. GBV-C/HGV has been suggested as important in the etiology of fulminant hepatitis [Yoshiba et al., 1995] and may be implicated in a significant number of acute and chronic cases of non A-C hepatitis [Fiordalisi et al., 1996]. However, insufficient data are available at

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present to reach conclusions about the pathogenicity of this agent [Alter, 1996].

Three isolates, GBV-C prototype from West Africa [Leary et al., 1996a] and two HGV isolates from the United States [Linnen et al., 1996], have been sequenced completely. It is not known if different genomic groups (genotypes) of GBV-C/HGV exist, as is the case for hepatitis B virus (HBV) and HCV. We evaluated the prevalence of the virus in (i) hemodialysis patients and (ii) non A-C hepatitis patients. We sequenced a segment of NS5 region from five Brazilian GBV-C/HGV isolates and compared the sequences to the viruses from West Africa and the United States.

MATERIALS AND METHODS

Clinical Specimens

Sera were obtained from blood samples collected in 1995 and 1996 and referred to the National Reference Center for Viral Hepatitis for serological analysis. Sixty-five patients were studied (41 men and 24 women, mean [±SD] age 47 ± 16 years) with chronic renal failure who were undergoing hemodialysis from two different dialysis centers in Rio de Janeiro, Brazil. The mean duration of hemodialysis was 64 ± 53 months. Twenty-seven serum samples were collected from sporadic cases of community-acquired non A-C hepatitis, 13 were from patients with hepatitis with at least one serological marker for HBV or HCV infection and 11 were from asymptomatic individuals with or without serological markers for infection by hepatitis B and C viruses.

Serology

Sera were tested for the presence of hepatitis B surface antigen (HBsAg) and for antibodies to the hepatitis B core antigen (anti-HBc) using Hepanostika HBsAg Uni-form II and Hepanostika anti-HBc Uniform, respectively, in microELISA system (Organon Teknika, The Netherlands) according to manufacturer's instructions. Anti-HCV antibodies were detected by in-house second generation enzyme linked immunosorbent assay (ELISA) with controlled specificity and sensitivity according to available commercial kits, and using recombinant antigens from core, NS3, and NS5 regions from the Research Foundation for Microbial Disease of Osaka University, Japan.

RNA Extraction and PCR

Total RNA was extracted from sera by the guanidinium thiocyanate method [Chomczynski and Sacchi, 1987] modified as follows: 100 μl serum was added to 900 μl of a mixture volume:volume of solution A (5.5 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% N-laurylsarcosine) and solution B (water-saturated phenol, 0.2 M sodium acetate, pH 4.0, 0.1 M 2-mercaptoethanol). After addition of 10 μg yeast tRNA, the mix was vortexed for 2 min, incubated for 30 min at room temperature, extracted with chloroform:isoamyl alcohol, and precipitated with isopropanol. The pellet was washed with 70% ethanol, air dried, and resuspended in 6 μl of a solution containing 5 U of RNase inhibitor and 1 μM random hexanucleotides (Life Technologies Inc. Gaithersburg, MD). After incubation at 65°C for 10 min, the entire RNA was reverse transcribed in a 21 μl reaction containing 50 mM Tris-HCl pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate (dNTP), and 200 U Moloney murine leukemia virus reverse transcriptase (Life Technologies Inc.). The mixture was incubated at 37°C for 1 hr, then at 65°C for 10 min.

One-third of the resultant cDNA was used in a PCR reaction (50 μl) with 0.4 μM of each of the primers. Sequences of the GBV-C/HGV NS5 region were detected after nested PCR using external primers YK874 (5′ CTGATGTTGCTAGCCTGTGTGAGA 3′, nt 7215 to 7238 on the GBV-C prototype genome) and YK877 (5′ ACCGACACCTTAGATCCCCAGCCC 3′, nt 7686 to 7663) and internal primers YK1183 (5′ CAGAACCATACAGCCTATTGTGAC 3′, nt 7247 to 7270) and YK876 (5′ CCTTACAGTCCTTATTGCTTCCTC 3′, nt 7648 to 7625) [Viazov et al., 1997]. HCV sequences were detected from the same cDNA by nested PCR using oligonucleotides from 5′-noncoding region: 5′ CACTCCCCTGTGAGGAACTACTGTC 3′, external sense, nt −304 to −280; 5′ TTCACGCAGAAAGCGTCTAGCC 3′, internal sense, nt −279 to −258; 5′ GGGCACTCGCAAGCACCCTATCAGG 3′, internal antisense, nt −26 to −50; 5′ ATGGTGCACGGTCTACGAGACCTCC 3′, external antisense, nt +3 to −23. After an initial denaturation of 2 min at 94°C, cDNA was amplified for 32 cycles at 94°C for 30 sec, 60°C (50°C for HCV) for 1 min, and 72°C for 1 min followed by a final elongation of 7 min at 72°C. One microliter of the product was subjected to nested PCR for 30 cycles in the same conditions. To avoid contamination, RNA extraction and reverse transcription, pre-PCR reagent preparation, DNA amplification, and gel electrophoresis of PCR products were performed in four separate rooms. No GBV-C/HGV nucleotide sequence being identical to each other, this allowed us to eliminate the possibility of cross-contamination.

Cloning and Nucleotide Sequencing

Nested PCR products (402 bp) from GBV-C/HGV NS5 region were quantified by ethidium bromide staining using DNA standards as controls and 5–10 ng was ligated into 50 ng pCRII vector (TA cloning kit; Invitrogen San Diego, CA). Recombinant plasmid DNA was purified for sequencing by ultracentrifugation in CsCl gradient. Nucleotide sequence of a 354 bp DNA fragment was determined by Cy5 autoread sequencing system (Pharmacia Biotech, Uppsala, Sweden) using M13 universal primers. Sequencing reactions were analyzed on an ALFexpress automated sequencer (Pharmacia).

Computer Analysis

Wilcoxon two-sample test was used to determine the statistical significance of differences between groups. Alignment of multiple nucleic acid sequences was performed with the University of Wisconsin Genetic Computer Group (GCG) program PILEUP. For the construction of a dendrogram, our sequences were com-

TABLE I. Presence of HBsAg, Anti-HBc, Anti-HCV, and GBV-HGV RNAs in Brazilian Serum Samples of Different Origins

Clinical data	n	Number of patients positive for			
		HBsAg	Anti-HBc	Anti-HCV	GBV-C/HGV RNA
Hemodialysis	65	12	34	25	10
Non A-C hepatitis	27	0	0	0	5
Hepatitis with HBV or HCV marker	13	1	6	8	2[a]
Asymptomatic[b]	11	0	2	7	2
Total	116	13	42	40	19

[a]Patients with chronic hepatitis, one HBV carrier and one HCV carrier.
[b]Among asymptomatic persons, four were individuals without serological markers for hepatitis A, B, and C virus infection and seven were anti-HCV positive (two of them anti-HBc positive). Of the four individuals without markers, two were health care workers (one of them GBV-C/HGV RNA positive).

TABLE II. Clinical and Serological Data of 19 Individuals Infected by GBV-C/HGV

Patient	Sex	Age (y)	Clinical data	HBsAg	Anti-HBc	Anti-HCV	HCV RNA	GBV-C/HGV RNA[b]
HD08-BR	M	74	Hemodialysis (149)[a]	–	+	+	–	+(2)
HD13-BR	M	18	Hemodialysis (52)	+	+	–	–	+(2)
HD19-BR	M	63	Hemodialysis (130)	+	+	+	–	+(2)
HD26-BR	F	32	Hemodialysis (198)	–	+	+	–	+(2)
HD27-BR	F	61	Hemodialysis (93)	–	–	+	–	+(2)
HD29-BR	M	50	Hemodialysis (4)	–	–	–	–	+(2)
HD40-BR	F	49	Hemodialysis (76)	–	+	+	–	+(2)
HD44-BR	F	23	Hemodialysis (18)	–	+	–	–	+(2)
HD48-BR	F	42	Hemodialysis (28)	–	–	–	–	+(2)
HD52-BR	M	35	Hemodialysis (11)	–	–	–	–	+(2)
S059-BR	F	Unknown	Acute hepatitis Non A-C	–	–	–	–	+(2)
S517-BR	M	46	Acute hepatitis Non A-C	–	–	–	–	+(2)
S545-BR	F	30	Chronic hepatitis Non A-C	–	–	–	–	+(2)
S794-BR	M	76	Chronic hepatitis Non A-C	–	–	–	–	+(2)
S956-BR	M	18	Acute hepatitis Non A-C	–	–	–	–	+(2)
B536-BR	M	32	Chronic hepatitis HBV carrier	+	+	–	–	+(1)
C737-BR	F	63	Chronic hepatitis HCV carrier	–	–	+	+	+(2)
C757-BR	F	43	Asymptomatic HCV carrier	–	+	+	+	+(2)
A001-BR	M	23	Asymptomatic	–	–	–	–	+(1)

[a]The duration (in months) of hemodialysis is indicated in parentheses.
[b]Amplicons were observed on agarose gels after the first (1) or only after the second (2) round of PCR.

pared with the three GBV-C/HGV complete genomes available in databanks of which the Genbank accession numbers are U36380 (GBV-C prototype), U44402 (HGV isolate PNF2161), and U45966 (HGV isolate R10291).

RESULTS
Detection of GBV-C/HGV RNA in Brazilian Patients

The 116 patients studied were divided in four groups: (i) patients on hemodialysis, (ii) non A-C hepatitis patients, (iii) patients with hepatitis that exhibited at least one serological marker for HBV or HCV infection, and (iv) asymptomatic individuals with or without serological markers for HBV and HCV infection (Table I). HBsAg, anti-HBc, and anti-HCV markers were present in 12 (18%), 34 (52%), and 25 (38%) patients on maintenance hemodialysis, respectively. Ten (15%) hemodialysis patients and 5 (19%) non A-C hepatitis patients were GBV-C/HGV RNA positive, along with one patient with chronic hepatitis B, one patient with chronic hepatitis C, and two asymptomatic individuals. No significant correlation was found between GBV-C/HGV RNA positivity and any of the following parameters: sex, age, HBsAg, and anti-HCV. In the hemodialysis group, the difference between the mean duration of dialysis of the GBV-C/HGV RNA positive (75.9 months) and negative (60.9 months) patients was not statistically significant. Table II shows clinical and serological data of the 19 GBV-C/HGV RNA positive individuals. GBV-C/HGV sequences were detected after the first round of PCR in two individuals (B536-BR and A001-BR) and only after nested PCR in the others. Three patients (HD13-BR and HD19-BR on hemodialysis and B536-BR with chronic hepatitis) were also HBsAg carriers and seven were anti-HCV positive. Serum of patient HD19-BR was at the same time positive for HBsAg, anti-HCV, and GBV-C/HGV RNA. Both HCV and

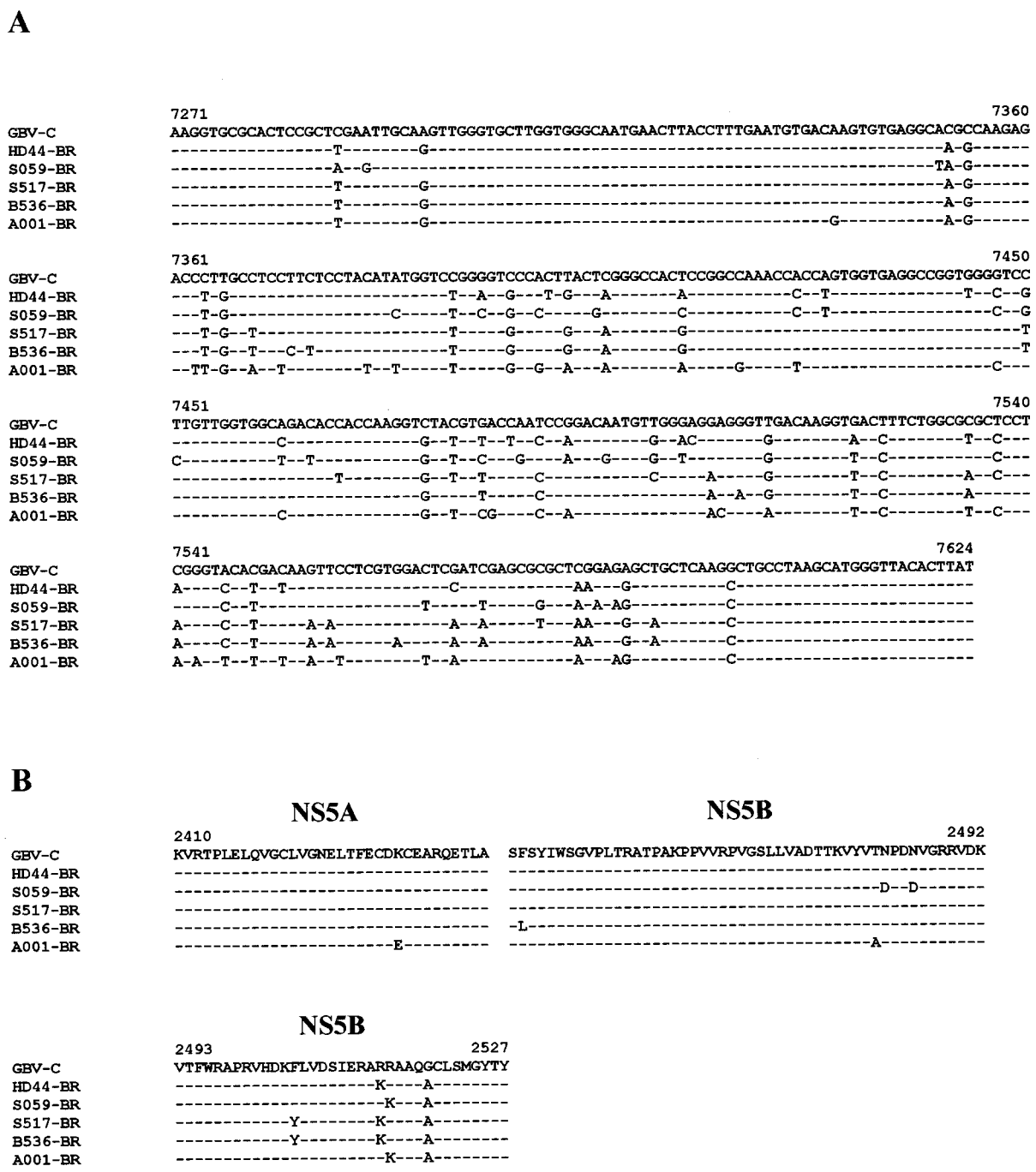

Fig. 1. Partial nucleotide **(A)** and deduced amino acid **(B)** sequences of Brazilian GBV-C/HGV isolates and comparison with the corresponding sequences of the GBV-C prototype (Genbank accession number U36380). Putative NS5A/NS5B cleavage site according to Leary et al. [1996a].

GBV-C/HGV RNAs were present in the sera of patients C737-BR and C757-BR.

Nucleotide and Amino Acid Sequence Comparison of GBV-C/HGV Isolates

The nucleotide sequences of five isolates, including at least one from each of the four populations, were determined over a 354 bp segment overlapping part of NS5A and NS5B regions. Figure 1A shows a comparison between the nucleotide sequences of our isolates and that of GBV-C prototype strain. No sequence was identical to each other. Seventy-five (21%) nucleotides were variable, that is mutated in at least one sample. The homologies between pairs of the five Brazilian sequences were between 87% and 97% (Table III). Homologies of 86–93% were observed when GBV-C/HGV strains from Brazil and from other countries [Leary et al., 1996a; Linnen et al., 1996] were compared. At the

TABLE III. Nucleotide Sequence Homologies Between Brazilian and Other GBV-C/HGV Samples (Partial NS5 Region)*

	Sequence homology, %							
	HD44-BR	S059-BR	S517-BR	B536-BR	A001-BR	U36380	U44402	U45966
HD44-BR	100	90	93	92	91	88	93	90
S059-BR		100	89	87	88	88	88	87
S517-BR			100	97	91	89	91	91
B536-BR				100	90	90	90	90
A001-BR					100	86	88	90
U36380						100	86 (86)	87 (86)
U44402							100	87 (91)
U45966								100

*In parentheses: percent of homology between entire genomes.

amino acid level, only nine (7%) residues differed between GBV-C prototype and our isolates (Fig. 1B). In all combinations, the level of homology among amino acid sequences was substantially higher (94.9% to 99.2%) than that observed at the nucleotide level (not shown). This was due to the fact that most substitutions occurred at the third nucleotide position of codons without changing the codon meaning. In particular, from nucleotides 7532 to 7558, GBV-C prototype and A001-BR strains presented at least one substitution in eight of nine codons, without any change in the deduced amino acid sequence (Fig. 1B; residues 2497 to 2505). In sample B536-BR, a mutation Phe to Leu was noted at position 2444, close to the putative NS5A/NS5B cleavage site. Phylogenetic analysis (Fig. 2) showed that strains S517-BR, B536-BR, HD44-BR, and U44402-USA were closely related and that the isolates S059-BR (Brazil) and U36380 (West Africa) were the most distant from the others.

DISCUSSION

GBV-A, GBV-B, and GBV-C/HGV are three recently discovered viruses which form, together with HCV, a discrete group in the family *Flaviviridae* [Harrison et al., 1996; Karayiannis and McGarvey, 1995; Zuckerman, 1996]. The presence of GBV-C/HGV has been detected in the blood of asymptomatic individuals and, at a higher prevalence, in patients with acute or chronic hepatitis of unknown etiology. The diagnostic of GBV-C/HGV infection has become possible by detection of viral RNA after RT-PCR using degenerated primers deduced from the putative helicase gene in the NS3 region [De Lamballerie et al., 1996; Fiordalisi et al., 1996; Masuko et al., 1996; Nübling and Löwer, 1996; Yoshiba et al., 1995] or consensus primers [Leary et al., 1996b]. Sequence alignments within 100 bp of NS3 region have allowed evaluation of the genetic heterogeneity of GBV-C/HGV isolates [Egawa et al., 1996; Kao et al., 1996; Simons et al., 1995a; Tsuda et al., 1996]. Using the degenerated primers G8, G9, and G11 [Yoshiba et al., 1995] of NS3 region, we noted the presence on agarose gels of several additional non-specific DNA bands in a number of samples (not shown). However, PCR assays with NS5 region target sequences gave only one product of expected size, facilitating the cloning experiments. The amplicon was larger (402 bp) than that obtained from NS3 region, reducing the statistical uncertainty of the sequence alignments. The homology rates between the three GBV-C/HGV isolates completely sequenced at this time [Leary et al., 1996a; Linnen et al., 1996] were similar when calculated from the entire genome or from part of NS5 region (Table III). It has been shown that HCV genotypes could be distinguished on the basis of the sequence identity of a small fragment of 222 nucleotides in the NS5B region [Simmonds et al., 1993]. Similarly, the NS5 region may be an appropriate genomic region to discriminate between different GBV-C/HGV subtypes.

Since GBV-C/HGV can be transmitted parenterally, we screened the presence of the virus in an at risk population of hemodialysis patients treated in the city of Rio de Janeiro, Brazil. GBV-C/HGV RNA was detected in sera of 10 (15%) patients. This positivity level was intermediate between the relatively low prevalence of 3.1% reported in Japan [Masuko et al., 1996] and the very high rates of 55% and 57.5% observed in Indonesia [Tsuda et al., 1996] and France [De Lamballerie et al., 1996], respectively, in hemodialysis patients. Five non A-C hepatitis patients, one chronic hepatitis B patient, one chronic hepatitis C patient, and two asymptomatic individuals were also positive. For non A-C hepatitis patients, our results showed a prevalence of 19%. This was higher than the numbers of 8.2% found in European samples and 12.5% noted in South America [Linnen et al., 1996], but lower than observed in Italy from non A-E acute and chronic hepatitis cases, 35% and 39%, respectively [Fiordalisi et al., 1996]. In addition, another study including a large number of sera from voluntary blood donors remains necessary to evaluate the GBV-C/HGV prevalence in the Brazilian general population.

Since little is known about the molecular epidemiology of GBV-C/HGV, diverse genotypes of this novel virus may await discovery. Recently, the existence of two different subtypes of GBV-C/HGV in Italy has been suggested [Fiordalisi et al., 1996] and 21 Taiwanese isolates have been classified into three groups [Kao et al., 1996]. To differentiate HCV genotypes, subtypes, and isolates, sequence identity values of 68–75%, 83–85%, and 92–98%, respectively, have been proposed [Shukla et al., 1995]. The level of homology between

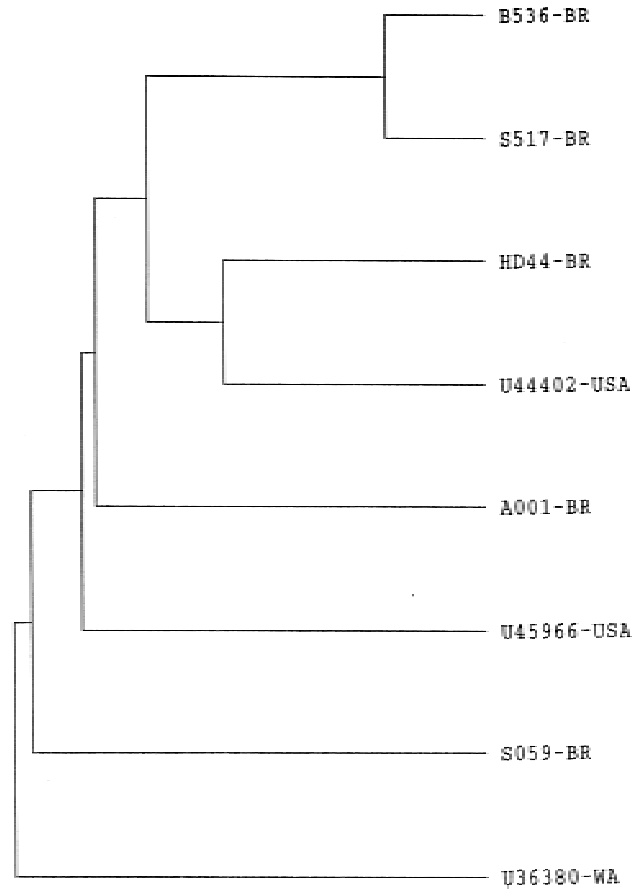

Fig. 2. Phylogenetic analysis of partial NS5 sequences of the GBV-C/HGV genome. Non-Brazilian isolates are referred by their Genbank accession number and their country of origin. BR: Brazil; WA: West Africa.

the nucleotide sequences of 5 Brazilian GBV-C/HGV strains was between 87% and 97%, and 86%–93% when our isolates were compared with the three ones previously sequenced [Leary et al., 1996a; Linnen et al., 1996]. The dendrogram of Figure 2 showing five main branches, along with the homology rates between sequences, may suggest that five subtypes are represented there. Sequencing a larger number of samples and other regions of the genome should help establish whether these different virus strains belong to the same genotype.

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